VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

The following two paragraphs have been inserted after the heading "INTRODUCTION" on page 1:

Cross-Reference to Related Applications

This application is a divisional of U.S. patent application serial no. 09/359,564, filed July 22, 1999.

The paragraph beginning on page 16, line 3 has been replaced with the following rewritten paragraph:

The above steps of contacting the ribonucleic acid with the polymerase and labeled ribonucleotide result in the production of an end-labeled ribonucleic acid characterized by the presence of one or more labeled ribonucleotide residues sequentially attached to the 3' terminus of the original ribonucleic acid via a phosphodiester linkage. The number of labeled ribonucleotide analogue residues that may be attached is at least 1, may be at least 2, where the number may be as high as 5 or 10 or higher[, but generally does not exceed about].

Vol. 41, No. 5, April 1997 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 1045–1050

IDENTIFICATION OF TWO POLY(A) POLYMERASES IN *BACILLUS SUBTILIS*

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Received January 9, 1997

SUMMARY Two poly(A) polymerase activities were identified in extracts of a strain of *Bacillus subtilis* in which the gene for polynucleotide phosphorylase was disrupted. Gel filtration studies showed a large difference in the molecular size of the two poly(A) polymerases. On the other hand, the two enzymes resembled the two major poly(A) polymerases of *Escherichia coli* both with respect to size and in many of their catalytic properties. The observation that both *B. subtilis* and *E. coli* have two poly(A) polymerases with many common properties suggest interesting parallels in the processing of the 3'-ends of mRNA in gram-positive and gram-negative bacteria.

Key Words: Poly(A) Polymerases, Bacterial Polyadenylation, *B. subtilis* Poly(A)

INTRODUCTION

Studies over the past 20 years on the polyadenylation of the 3'-ends of bacterial mRNA have shown that this type of mRNA processing is a general phenomenon in prokaryotes as well as in eukaryotes (1). The case for mRNA polyadenylation is especially clear in *E. coli*, where the nucleotide sequences at the polyadenylation sites of several mRNAs have been determined (2-3; reviewed in ref. 4) and two poly(A) polymerases have been identified (5) and their genes cloned (6, 7). Although the earliest detailed characterization of polyadenylated bacterial mRNA was in a *Bacillus* species (8) and the first bacterial cDNA library was prepared from *Bacillus subtilis* poly(A) RNA (9, 10), work on *B. subtilis* poly(A) RNA has lagged behind that in *E. coli*. A nucleotide sequence homologous to *pcnB*, the gene for the major *E. coli* polymerase, has been identified on the *B. subtilis* chromosome (11) but no poly(A) polymerase activities have yet been described in *B. subtilis* extracts. In this paper, we report the presence of two poly(A) polymerase activities in *B. subtilis*, which correspond in their molecular size and relative amounts to the two major poly(A) polymerases of *E. coli*.

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1039-9712/97/051045-06$05.00/0

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions: *Bacillus subtilis* BD2256 [*pnpA*::mini-Tn10(*cat*) *amyE*::*comG-lacZ*(*kam*) (12)] was a gift from Dr. D. Dubnau. *B. subtilis* 168 was the wild-type laboratory strain. Bacteria were grown at 37 °C on a rotary shaker in LB supplemented with chloramphenicol (4 µg/ml). They were harvested in mid-exponential phase by centrifugation and stored at -70 °C.

Poly(A) Polymerase Assay: The assay mixtures (50 µl) contained 50 mM Tris·HCl, pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 10 mM creatine phosphate, 2 µg creatine phosphokinase, 3 µM rifampicin, 25 µg gelatin, 10 µg of poly(A), and 0.12 mM [α-^{32}P] ATP (100 µCi/µmol). After incubation at 37 °C for 10 min, incorporation of radioactivity into poly(A) was determined by adsorption on DE-81 filter discs as described (5). One unit of poly(A) polymerase activity is defined as the amount of enzyme that incorporates 1 nmol of ATP in 10 min at 37 °C.

Separation of Poly(A) Polymerase Activities by Gel Filtration: Cells (1.2 g) of *B. subtilis* BD2256 were suspended in 3 ml of Buffer E (50 mM Tris·HCl, pH 8.0, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM p-toluene sulfonyl fluoride, 3 µM leupeptin, 2 µM pepstatin, 1 µg/ml pancreatic DNase, 5 % glycerol) and disrupted in a French pressure cell at 4 °C. The crude extract was centrifuged at 10,000 x g for 10 min and the supernatant was adjusted to 1.0 M by adding solid NH_4Cl, followed by centrifugation at 150,000 x g for 1 h. A portion (2 ml) of the ribosome-free supernatant fraction was loaded on a 100-ml Sephacryl S200 column equilibrated with Buffer S (10 mM Tris·HCl, pH 8.0, 0.5 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20 % ethylene glycol), and fractions (2 ml) were collected and assayed for poly(A) polymerase activity. The concentration of protein in the fractions was determined by the method of Bradford (13).

RESULTS

Poly(A) polymerase activity in the ribosome-free supernatant fraction of extracts of *B. subtilis* 168 was found to be about 20 % of the activity level (9 units per mg of protein) in comparable *E. coli* extracts (6). When the crude *B. subtilis* extracts were supplemented with 1 M NH_4Cl prior to sedimentation of the ribosomes, about twice the amount of poly(A) polymerase activity was recovered in the ribosome-free supernatant, suggesting significant salt-dependent association with ribosomes analogous to that observed in *E. coli* (6). More detailed studies were carried out with extracts of *B. subtilis* BD2256, a strain which lacks polynucleotide phosphorylase, an enzyme which can polymerase ADP and potentially mimic poly(A) polymerase activity when the labeled ATP substrate is partially degraded to ADP by endogenous ATPase activities. The specific activity of poly(A) polymerase in ribosome-free extracts of this strain, prepared in the presence of 1 M NH_4Cl, was 2 units per mg of protein.

Assay of poly(A) polymerase activity in the ribosome-free fraction of *B. subtilis* BD2256 showed an absolute requirement for divalent cations, with 2 mM Mn^{2+} more effective than 10 mM Mg^{2+} but less effective than a combination of both, similar to the response of the major poly(A) polymerase of *E. coli* (6). Rifampicin and streptolydigin, which are potent inhibitors of bacterial

RNA polymerases, had no effect on *B. subtilis* poly(A) polymerase. Inorganic phosphate (0.5 mM), which strongly inhibits the polymerizing activity of polynucleotide phosphorylase, and cordycepin triphosphate (100 μM), an inhibitor of eukaryotic poly(A) polymerases, also had no effect. Spermidine (5 mM) slightly stimulated (12 %) poly(A) polymerase activity. Poly(A) polymerase activity required the presence of an RNA primer, with poly(A), dephosphorylated poly(A), tRNA all being effective.

When the ribosome-free supernatant fraction of *B. subtilis* BD2256 was subjected to gel filtration on a column of Sephacryl S200 using a high-salt buffer (0.5 M KCl) to prevent protein aggregation, two peaks of poly(A) polymerase activity were observed. The first peak, which accounted for 90 % of the recovered poly(A) polymerase, eluted with K_d of 0.11, whereas the second peak (10 % of the recovered activity) eluted with K_d of 0.40 (Fig. 1). Comparison with the elution positions of ovalbumin (K_d of 0.33) and lysozyme (K_d of 0.73) on the same Sephacryl S200 suggested molecular weights of about 86,000 and 37,000 for the major and minor poly(A) polymerases of *B. subtilis*, assuming that these proteins and the molecular weight standards have similar molecular shapes. When poly(A) polymerase activity was measured using tRNA rather than poly(A) as primer, a third peak with K_d of about 0.47 was observed, which overlapped with the second poly(A) polymerase fraction (data not shown) and probably represented tRNA nucleotidyl transferase.

Some of the catalytic properties of the two poly(A) polymerase fractions (Peak I and Peak II) are summarized in Table 1. Although the properties of the two enzymes were qualitatively similar with respect to relatively high apparent K_m's for ATP, susceptibility to inhibition by dATP and other ribonucleoside triphosphates, inhibition by 1,10-phenanthroline, and stimulation by KCl at intermediate concentrations and inhibition by high concentrations of KCl, there were significant quantitative differences with respect to these parameters.

DISCUSSION

This paper reports the separation and preliminary characterization of two poly(A) polymerase activities from *B. subtilis*. In many respects, the properties of these enzyme fractions were quite similar to the two major poly(A) polymerases of *E. coli*, PAPI and PAPII (5-7). Most striking in this respect were the relative elution patterns from Sephacryl S200, with K_d's of 0.11 and 0.40 for the *B. subtilis* enzymes and of 0.12 and 0.44 for those from *E. coli*, as well as the relative amounts of the two enzyme forms, the larger protein accounting for 90% or more of poly(A) polymerase activity under the growth and assay conditions employed. Other similarities were the requirement for both Mn^{2+} and Mg^{2+} and intermediate salt concentrations for optimal poly(A) polymerase activity and inhibition by high salt and dATP. It is therefore tempting to speculate that poly(A) polymerase Peak I and Peak II of *B. subtilis* are homologous to PAPI and PAPII of *E. coli*, although direct support for this hypothesis must await a molecular

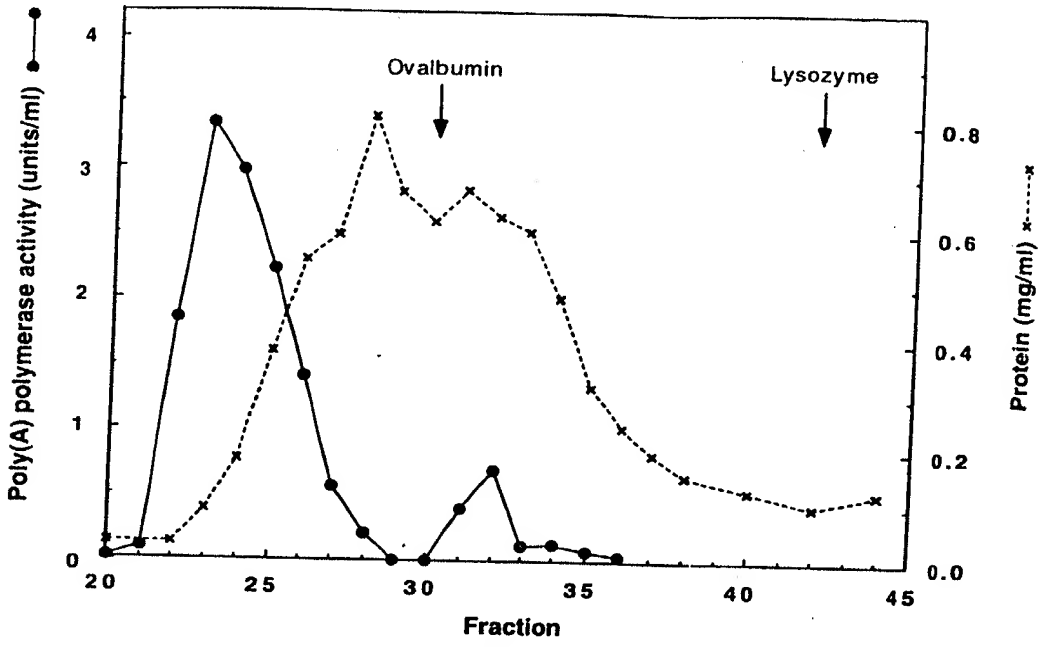

Fig. 1. Gel filtration on Sephacryl S-200 of poly(A) polymerase activity from *B. subtilis* BD2256 *pnpA*::mini Tn10 (*cat*). Poly(A) polymerase activity was fractionated from ribosome-free supernatant (see Methods). Two ml supernatant was passed through a column (1 cm x 127 cm) of Sephacryl S-200 in a buffer containing 10 mM Tris HCl, (pH 8.0), 0.5 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 20% ethylene glycol. Fractions (2.0 ml) were collected and assayed for poly(A) polymerase activity using poly(A) as primer. The *arrows* indicate the elution positions of ovalbumin and lysozyme on the same column.

TABLE 1

Comparison of the Properties of *B. subtilis* Poly(A) Polymerase Peak I and Peak II

Property	Peak I	Peak II
Apparent K_m for ATP	400 μM	143 μM
Inhibition by 250 μM dATP	50 %	15 %
Inhibition by 80 μM each of GTP, CTP and UTP	47 %	30 %
Inhibition by 160 μM each of GTP, CTP and UTP	63 %	46 %
Inhibition by 1 mM 1,10-phenanthroline	28 %	50 %
Optimal KCl concentration	250 mM	125 mM
Inhibition by 300 mM KCl	15 %	52 %
Inhibition by 500 mM KCl	100 %	100 %

characterization of the proteins and their genes. A nucleotide sequence with significant homology to *pcnB*, the *E. coli* gene encoding PAPI, has been identified on the *B. subtilis* chromosome (11) and Western blot analysis of *B. subtilis* extracts with antibodies against *E. coli* PAPI has revealed the presence of a cross-reacting protein (14). On the other hand, a nucleotide sequence homologous to *f310*, the *E. coli* gene encoding PAPII, has not yet been reported in *B. subtilis*.

The discovery of two poly(A) polymerases in *B. subtilis* with significant functional similarities to the two major *E. coli* poly(A) polymerases suggests interesting parallels in the processing of the 3'-ends of mRNA between Gram-positive and Gram-negative bacteria. Earlier analysis of the site of polyadenylation of the mRNA encoded by the *B. subtilis hag* gene (15) showed that it corresponded to Class II poly(A) RNA, the predominant form of polyadenylated RNA in *E. coli*. Class II poly(A) RNA is probably the result of polyadenylation following RNase E cleavage of the terminal stem-loop structure of the primary transcript (1, 2), and its occurrence in both *B. subtilis* and *E. coli* suggests similarities in the dynamics of mRNA degradation and polyadenylation. Indeed, the major 3'-exonucleases responsible for the degradation of mRNA 3'-termini in *E. coli*, ribonuclease II and polynucleotide phosphorylase, have counterparts in the K^+-dependent 3'-exoribonuclease of *Bacillus brevis* (16) and *B. subtilis* (17) and the polynucleotide phosphorylase encoded by the *pnpA* gene of *B. subtilis* (18, 19), respectively, although the relative contributions of hydrolytic and phosphorolytic exonuclease digestion of mRNA may differ in *E. coli* and *B. subtilis* (20-22).

When the present identification of two poly(A) polymerases is confirmed at the molecular level, all the major enzymes involved in the processing of the 3'-ends of mRNA in *B. subtilis* would be known except for the counterpart of RNase E. This paves the way for the identification of the corresponding genes and the systematic study of their function by examining the effect of mutation and gene disruption on mRNA metabolism, analogous to the studies now in progress in *E. coli* (e.g. ref. 4). It also opens interesting new perspectives on the evolution of mRNA polyadenylation, for until now *E. coli* has been the only organism in which two poly(A) polymerases have been identified (7).

ACKNOWLEDGMENTS

We thank Dr. Henry Paulus for stimulating discussions and critical reading of the manuscript. This work was supported by National Institues of Health Grant R01-GM26517.

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